Vaccination of Colorectal Cancer Patients with Modified Vaccinia Ankara Delivering the Tumor Antigen 5T4 (TroVax) Induces Immune Responses which Correlate with Disease Control: A Phase I/II Trial

Richard Harrop,[1] Noel Connolly,[2] Irina Redchenko,[1] Juan Valle,[2] Mark Saunders,[2] Matthew G. Ryan,[1] Kevin A. Myers,[1] Noel Drury,[1] Susan M. Kingsman,[1] Robert E. Hawkins,[2] and Miles W. Carroll[1]

Abstract **Purpose:** The highly attenuated strain of vaccinia virus, modified vaccinia Ankara (MVA), encoding the tumor antigen 5T4 (termed TroVax), has been evaluated in an open-label phase I/II study in colorectal cancer patients. The primary objectives were to assess the safety and immunogenicity of ascending doses of TroVax and to determine the biodistribution of the vector.

Experimental Design: TroVax was given to 22 patients with metastatic colorectal cancer. Seventeen patients received doses of TroVax ranging from 5×10^7 up to 5×10^8 plaque-forming units at 0, 4, and 8 weeks and were considered to be evaluable for assessment of immunologic responses. Both antibody and cellular responses specific for the tumor antigen 5T4 and the viral vector were monitored throughout the study.

Results: TroVax was well tolerated in all patients with no serious adverse events attributed to vaccination. Of 17 evaluable patients, 16 showed 5T4-specific cellular responses whereas 14 had detectable antibody levels following vaccination. TroVax was able to boost 5T4-specific immune responses in the presence of MVA neutralizing antibodies. Periods of disease stabilization ranging from 3 to 18 months were observed in five patients, all of whom mounted 5T4-specific immune responses. Furthermore, statistical analysis showed a positive association between the development of a 5T4 (but not MVA) antibody response and patient survival or time to disease progression.

Conclusion: These data indicate that vaccination with TroVax is safe and well tolerated and that immune responses to 5T4 can be induced without any evidence of autoimmune toxicity. Furthermore, 5T4-specific antibody responses correlate with evidence of disease control.

Over the last decade, targeted immunotherapy approaches for the treatment of cancer have been investigated with renewed vigor, perhaps catalyzed by the clinical successes seen with monoclonal antibody–based therapies (1). Cancer immunotherapy, using a classic vaccine approach, is dependent on the induction of an immune response which is capable of killing tumor cells but inducing no, or limited, deleterious autoimmune reactions. The identification of a tumor-associated antigen which is expressed on the tumor target (both primary and metastases) but which is absent on all (or at least most) normal tissues is important for safe and targeted immunotherapy. The human oncofetal antigen 5T4 is a 72-kDa leucine-rich membrane glycoprotein which is expressed at high levels on the placenta and also on a wide range of human carcinomas including colorectal, gastric, renal, and ovarian cancers but rarely on normal tissues (2–4). Overexpression of 5T4 is associated with poor prognosis in patients with colorectal, gastric, and ovarian carcinoma (5). Additionally, when tumor cells are transfected with 5T4 cDNA, they display increased motility, suggesting that expression of this molecule may induce metastatic properties in a tumor (6, 7). The restricted expression of 5T4 on normal tissues and its high prevalence on many common human carcinomas make 5T4 an attractive target for cancer immunotherapy. Furthermore, its surface expression means that it could potentially be a target for both CTL and antibody-mediated effector responses.

In addition to the tumor-associated antigen, the antigen delivery system is equally as important for the development of a successful cancer vaccine. One possible way to induce a potent and targeted antitumor response is to use viruses to deliver the tumor-associated antigen to cells of the immune system. Because the first recombinant vaccinia virus was constructed more than two decades ago (8, 9), poxviruses such as vaccinia,

Authors' Affiliations: [1]Oxford BioMedica (UK) Ltd., The Medawar Centre, Oxford Science Park, Oxford, United Kingdom and [2]The Christie Hospital, Withington, Manchester, United Kingdom

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Note: Current address for Miles W. Carroll: MNI, pharma Ltd., University of Reading Science and Technology Centre, Reading, Berks RG6 6AH, United Kingdom.

Requests for reprints: Richard Harrop, Oxford BioMedica (UK) Ltd., The Medawar Centre, Oxford Science Park, Oxford OX4 4GA, United Kingdom. Phone: 44-1865-783000; Fax: 44-1865-783001; E-mail: r.harrop@oxfordbiomedica.co.uk.

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fowlpox, and canarypox have found widespread use as vaccine vectors in infectious disease and cancer research (the latter reviewed in ref. 10) due to their good safety profile and efficient induction of both cellular and humoral immune responses. The safety profile of replication competent vaccinia virus was further improved by the generation of attenuated strains such as modified vaccinia Ankara (MVA; ref. 11). A number of tumor-associated antigens have been engineered into vaccinia virus vectors (including MVA) and the recombinant vaccines shown to induce tumor-associated antigen specific immune responses in cancer patients (12–14). Due to its documented safety profile and ability to induce potent immune responses, MVA was selected as the viral vector of choice to deliver the 5T4 tumor antigen to cells of the immune system. Prior preclinical studies showed that vaccination of mice with TroVax was safe and effective in both prophylactic and active treatment settings against a murine tumor cell line expressing 5T4 (15, 16). In this murine tumor model, protection was $CD4^+$ T cell dependent and antibody mediated (16).

Colorectal carcinoma is one of the most common neoplasms in Western societies, being second only to lung cancer as a cause of death from malignancy. Typically, 50% to 70% of patients will undergo potentially curative surgery for colorectal cancer, and of these ~10% to 25% will develop local recurrence. Between 80% and 90% of patients with local recurrence are expected to die within 5 years. Although treatment for metastatic disease has improved in recent years, it remains palliative and may have considerable toxicity (17). Given the high level of 5T4 expression on colorectal tumors and its correlation with poor prognosis, and the lack of effective alternative treatments for patients with stage IV disease, TroVax represents a potential novel therapeutic option. This phase I/II clinical trial represents the first time that TroVax has been given to patients. Here we report details of the safety and immunogenicity of ascending doses of TroVax delivered to colorectal cancer patients via intramuscular (i.m.) or intradermal (i.d.) route.

Patients and Methods

Patients. This phase I/II trial was an open-label upward titration study of TroVax given to patients via i.m. injection. In addition, a single dose level of TroVax, administered i.d., was included to explore the potential effect of this route on safety and immunogenicity. Adult patients (>18 years old) with metastatic colorectal cancer, who had responded to or stabilized on first-line chemotherapy, were eligible for inclusion into this study. This cohort was selected for an immunotherapy approach because patients with rapid disease progression would be eligible for immediate second-line chemotherapy and would also be less likely to benefit from an immune response which took a month or more to mature. Additional inclusion criteria included a minimum of 2.5 months since completion of chemotherapy and evaluation of general immunocompetence by determination of total white cell count and CD4/CD8 T-cell ratio. All patients had a WHO performance status of 0, 1, or 2 and were expected to survive for >3 months. The trial protocol was reviewed and approved by the Gene Therapy Advisory Committee and the study conducted under a Clinical Trial Exemption granted by the Medicines Control Agency. The trial was approved by the South Manchester Local Research Ethics Committee before commencement of enrollment and informed consent was obtained from each patient before enrollment.

Vaccine composition. TroVax was produced by the homologous recombination of human 5T4 cDNA into deletion region III of MVA and placed under the control of the modified H5 promoter (16). Subsequently, clinical grade material was manufactured and vialled under good manufacturing practice conditions (IDT, Rosslau, Germany).

Clinical trial design. At the time of entry to the trial, each patient underwent chest, abdominal, and pelvic computer-assisted tomography (CT) scans to quantify the extent of tumor metastases. In addition, the concentration of the surrogate marker, carcinoembryonic antigen (CEA), was measured in the plasma. Patients were assigned sequentially to four groups commencing at the lowest vaccine dose. Group 1 received $\sim 5 \times 10^7$ plaque-forming units (pfu; 1×; patients 101-104), group 2 received 2.5×10^8 pfu (5×; patients 201-205), and group 3 received 5×10^8 pfu (10×; patients 301-308) TroVax via i.m. injection in a volume of 1 mL into the deltoid muscle. Group 4 received 1×10^8 pfu (2×; patients 401-407) TroVax via the i.d. route in a volume of 100 μL using a BioJector 2000 needle-free injection management system (BioJect, Inc., Portland, OR). Patients received a series of three injections at weeks 0, 4, and 8 (Fig. 1). If a clinical response was detected by week 12 or an immunologic response detected between weeks 0 and 12, patients were offered a further two injections at approximately weeks 14 and 20. Blood samples were taken for immunomonitoring and blood biochemistry throughout the trial. Safety was evaluated in terms of adverse events graded according to common toxicity criteria, laboratory tests, and vital signs.

Biodistribution of the vector in the periphery was monitored by quantitative PCR (ABI Prism 7700, Perkin-Elmer, Beaconsfield, Bucks, United Kingdom). Following the first TroVax injection, blood samples were obtained at 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours, and 2 weeks postimmunization. Following subsequent injections, blood samples were obtained at 2 hours postimmunization only. In addition, the presence of any residual virus at the injection site was monitored by swabbing 2 hours postimmunization and doing plaque assays.

Processing of blood for immunomonitoring. All test articles (heparinized blood) were processed on the day of sampling by centrifugation through Histopaque columns (Accuspin, Sigma, Poole, Dorset, United Kingdom) to yield peripheral blood mononuclear cells and plasma. Peripheral blood mononuclear cells were used fresh in proliferation assays and any remaining cells were frozen under liquid nitrogen. All plasma was stored in aliquots at −80°C.

Antigens. Purified recombinant 5T4 protein (16) was used to monitor antibody responses (by ELISA and Western blot analysis) and cellular responses (by proliferation assay). In addition, a set of overlapping 20-mer peptides (Mimotopes, Clayton, Victoria, Australia) spanning the entire 5T4 amino acid sequence was used to measure cellular responses. Immune responses to additional antigens were also monitored throughout the trial; these included MVA and CEA (Merck Biosciences, Beeston, Nottingham, United Kingdom).

Measurement of humoral responses. ELISA was used to measure 5T4-, CEA-, and MVA-specific antibody titers as previously described (18). Antibody titer was defined as the greatest dilution of plasma at which the mean absorbance of the test plasma was ≥2-fold the mean absorbance of the negative control (normal human serum) at the same dilution. If a preexisting antibody response was detected, a positive

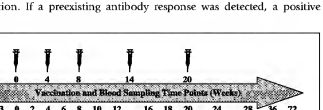

Fig. 1. TVI sampling schedule. The schematic illustrates each vaccination time point (syringe) and, below the solid arrow, time points at which blood samples were taken for monitoring of immune responses and CT scans were done to monitor disease progression.

response due to vaccination was reported if the postinjection antibody titer was ≥2-fold the antibody titer determined before TroVax immunization. In addition, MVA neutralizing antibody levels were determined as detailed in Harrop et al. (19).

Measurement of cellular responses. Proliferative responses were measured following stimulation with medium alone (RPMI/10; negative control), phytohemagglutinin (positive control), or test antigens (5T4 protein, 5T4 peptides, MVA, and CEA) as detailed in Harrop et al. (19). All protein antigens were used at a final concentration of 5 μg/mL, peptides at 4 μg/mL, and UV-inactivated MVA at 5×10^6 pfu/mL. A stimulation index ≥2 was considered to be positive. In addition, a positive response due to vaccination was defined as a stimulation index which was ≥2 and which was also ≥2-fold greater than the highest stimulation index induced by the antigen at either of the preinjection time points.

Clinical monitoring. Assessment of disease status was done by CT scanning (abdominal, pelvic, and/or pulmonary) using standard WHO criteria. In addition to CT scan analysis, the circulating levels of the surrogate markers CEA and CA-242 were measured in the plasma of patients at each sampling time point. Assays were done according to the instructions of the manufacturer (Alpha Diagnostic, San Antonio, TX; and CanAg, Gothenburg, Sweden, respectively). Results are reported as nanograms per milliliter of plasma (CEA) or units per milliliter of plasma (CA-242).

Statistical analysis. No formal statistical analysis of the data was planned for this phase I/II open-label study. However, a retrospective statistical analysis was undertaken, which aimed to identify potential correlations with patient survival and time to progression. A number of variables were investigated and included age of patient, sex, tumor burden, circulating CEA levels at trial entry, and antibody responses to MVA and 5T4 induced following vaccination. All seventeen evaluable patients were included in the statistical analysis. The duration of survival was defined as the whole number of months from the first TroVax immunization to the month of death or last known to be alive. Time to disease progression was calculated according to WHO criteria from the baseline CT scan taken before TroVax immunization. Cox regression analysis was used to model relative risk as a function of the predictors. A patient who died before evidence of progression was counted as progressing at the date of death. All statistical analyses were done with "S-plus" (version 6.2).

Results

Patients. In total, 22 patients were enrolled in the trial. Of those patients receiving TroVax via the i.m. route, four patients received the 5×10^7 pfu (1×) dose, five patients received the 2.5×10^8 pfu (5×) dose, and seven patients received the 5×10^8 pfu dose (10×). Six patients received 1×10^8 pfu (2×) TroVax via the i.d. route. All 22 patients were included in the intention-to-treat population, 5 patients withdrew before becoming evaluable, and 17 patients were included in the per-protocol population (received ≥3 TroVax immunizations) and were considered to be evaluable for assessment of immunologic and clinical responses. The mean (±SD) age of the entire group was 58.9 (±9.86 years; range, 39-75 years). All four treatment groups were broadly similar with mean (±SD) ages of 59.5 (±9.68), 53.6 (±13.24), 57.1 (±7.47), and 64.8 (±8.35) years for the 1×, 5×, 10×, and 2× groups, respectively. All patients conformed to WHO performance status 0 or 1 with the exception of one patient in the 10× group who had a performance status of 2. The characteristics of the intention-to-treat patient group are detailed in Table 1.

Safety. The most commonly reported treatment-related adverse events were nonserious, local events relating to the TroVax injection site. There was a higher incidence of injection site erythema in the i.d. injection group (3 patients; 50%). None of the adverse events leading to withdrawal were considered related to the study medication. A total of 10 patients reported serious adverse events (one in the 1× i.m. group, two in the 5× i.m. group, six in the 10× i.m. group, and one in the 2× i.d. group), all of which were related to the disease, not TroVax.

Biodistribution of the vector and persistence at the injection site. Blood samples were obtained at baseline and 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours, and 2 weeks after the first TroVax injection. Vector was not detected in any of these samples by quantitative PCR (data not shown). Residual vector was detected sporadically at the injection site in some patients (one patient in the 1× group, three patients in the 10× group, and three patients in the 2× group). However, the levels of vector detected were very low (<5 pfu; data not shown) and could be cleared by swabbing with ethanol.

TroVax induced antibody responses. 5T4-specific antibody responses were monitored by ELISA at each sampling time point and expressed as a titer compared with the negative control plasma (Table 2). No patient had a detectable 5T4-specific antibody titer before TroVax immunization and three patients (101, 201, and 202) failed to show an increase in antibody titer following vaccination. However, 14 patients showed detectable 5T4-specific antibody titers (ranging from 10 to 320), which were detectable after two or more vaccinations. The 5T4 specificity of the antibody response was confirmed by Western blot analysis for a number of these patients (data not shown). One patient (102) mounted a 5T4-specific antibody response, which was coincident with the detection of tumor necrosis and a reduction in the level of the circulating surrogate marker CA-242 (Fig. 2A). Furthermore, this patient mounted a potent CEA-specific antibody response, which corresponded with a dramatic decrease in the circulating levels of CEA detected in the plasma (Fig. 2B). The CEA antibody response was detected after the patient had received three TroVax immunizations and following the induction of a 5T4-specific antibody response.

MVA-specific antibody responses induced following vaccination with TroVax were monitored at weeks 0, 4, 8, 12, 24, and 52 by ELISA and compared with a pool of sera from five healthy vaccinia naïve donors. Table 3 summarizes the MVA-specific antibody titers in all 17 evaluable patients. Dilutions of plasma started at 1:4,000; therefore, samples which were not positive at this dilution are tabulated as having a titer of <4,000. Two patients (202 and 406) showed no evidence of sero-conversion at this dilution. Three patients (101, 204, and 402) had preexisting antibody titers to MVA, one of which (101) showed no further increase following vaccination. In total, 14 patients showed a positive MVA-specific antibody response following TroVax immunization, with titers ranging from 4,000 to 128,000. The majority of these patients sero-converted after a single TroVax immunization. The magnitude of the neutralizing antibody levels observed in all evaluable patients is tabulated alongside the total MVA-specific immunoglobulin G titers (Table 3). Four time points (0, 4, 8, and 12 weeks post primary immunization) were selected to measure the neutralizing antibody levels so that the effect of one, two, and three injections could be assessed. The neutralizing antibody titer was defined as the lowest serum dilution at which the number of foci was reduced by ≥50%. All patients showed neutralizing antibody titers ranging from 20 to >500. The neutralizing titers

Table 1. Intention to treat patient characteristics

Patient no.	Age (y)	Sex	Prior radiotherapy/chemotherapy	Metastatic disease	WHO status	No. vaccinations	Time on trial (wk)
101	68	M	Intrahepatic 5-FU/FA	Liver, LN	1	3	49
102	55	F	5-FU/FA ×6, Tomudex ×9, XRT	Mesenteric mass	0	4	34
103	67	M	Pre op XRT, dG ×12	Lung, liver	1	5	37
104	48	M	Radio frequency ablation	Lung, liver, LN	0	4	41
201	52	M	Post-op XRT, IrMdG ×6	Lung, liver; pre-sacral mass	0	3	14
202	57	M	IrMdG ×12	Liver, omental and serosal; left pleural effusion	0	3	13
203	39	M	IrMdG ×12	Lung, liver	0	1	0
204	46	F	Adjuvant 5-FU/FA	Mediastinal lymphadenopathy, lung, bone	0	5	72
205	74	M	IrMdG ×12	Liver, LN, mesenteric masses	1	5	72
301	49	M	IrMdG ×12	Lung, liver	0	2	6
302	48	M	IrMdG ×12	Lung, liver	1	2	6
303	68	M	Pre op XRT, IrMdG ×12	Large omental disease, LN	1	3	17
304	62	F	MdG ×12	Lung	1	1	4
305	55	F	Pre-op XRT, adjuvant 5-FU/FA, IrMdG ×6	Bone, LN	0	5	25
307	55	F	Post-op XRT, 5-FU/FA, IrMdG ×12 Pelvic XRT	Lung, liver, LN	2	4	17
308	63	M	Pre op XRT, OxMdG ×12	Liver	0	5	28
401	50	F	5-FU/FU, IrMdG	Lung	1	5	52
402	63	M	OxMdG ×1	Lung, liver, LN	1	5	20
404	75	M	MdG ×12	Liver	1	5	52
405	69	M	Capecitabine ×3	Lung, liver, bone, LN	1	1	4
406	65	M	IrMdG ×9	Liver, LN	0	3	12
407	67	M	5-FU/FA, IrMdG ×12	Liver	0	4	16

NOTE: The table details the dosage group, age, sex, prior therapy, the main sites of metastatic disease, WHO performance status, total number of TroVax injections received, and the time each patient remained on the trial (until death, withdrawal or end of the trial) following the primary immunization.
Abbreviations: 5-FU/FA, 5-fluorouracil and folinic acid; dG, deGramont regimen; IrMdG, Irinotecan modified de Gramont regimen; OxMdG, oxaliplatin modified deGramont regimen; XRT, radiotherapy; LN, lymph node.

correlated with the total MVA immunoglobulin G antibody response ($P < 0.01$) and were greatest in the 10× i.m. and 2× i.d. groups.

TroVax induced cellular responses. To monitor the induction of 5T4-specific cellular responses, a proliferation assay was done at each sampling time point on fresh peripheral blood mononuclear cells. Table 4 details the 5T4-specific proliferative responses, expressed as stimulation indices, for all per protocol patients. Four patients (101, 205, 303, and 307) showed evidence of a preexisting 5T4-specific proliferative response. However, following TroVax immunization, patient 205 showed a >2-fold increase in response compared with the 0 week preinjection time point. Following vaccination with TroVax, eight patients showed *de novo* 5T4-specific proliferative responses whereas six patients (101, 201, 202, 303, 401, and 406) failed to show positive proliferative responses. However, with the exception of two patients (101 and 303), the remaining four patients responded to one or more 5T4 20-mer peptides following, but not before, vaccination with TroVax (data not shown). The mean number of weeks before a positive proliferative response to 5T4 protein was detected was 8.7 weeks for the 1× i.m. group (range, 8-10 weeks), 9 weeks for the 5× i.m. group (range, 8-10 weeks), 3 weeks for the 10× i.m. group (2-4 weeks), and 7.3 weeks for the 2× i.d. group (range, 4-12 weeks). The overall mean time for a positive 5T4 proliferative response to be detected was 7.2 weeks (i.e., following 2 TroVax immunizations).

Clinical responses. Disease progression was monitored throughout the trial by determining the levels of the surrogate markers CEA and CA-242. In addition, CT scans were done at regular intervals to enable changes in tumor size or architecture to be detected. Five patients showed periods of disease stabilization (104, 204, 205, 401, and 404) ranging from 3 to 18 months. In general, increases in tumor burden accompanied elevated CEA levels (data not shown). One major exception was patient 102 who showed a 227% increase in tumor burden from the baseline to 3-month time point but a 93% decrease in the CEA levels. This observation could be a result of the induction of a CEA-specific antibody response in this patient, which may prevent the quantification of all the circulating CEA in the plasma by ELISA-based methodologies. The observed immunologic and clinical responses in all 17 evaluable patients are summarized in Table 5.

Correlations with time to disease progression and overall patient survival. A retrospective analysis of potential immunologic correlations with time to disease progression and overall patient survival was undertaken. Two of the variables analyzed were the MVA- and 5T4-specific antibody responses. Because all evaluable patients remained on the trial for different lengths of time, the mean antibody titer detected within the

first 12 weeks post primary immunization was calculated (i.e., the sum of the antibody titers detected at weeks 2, 4, 6, 8, 10, and 12 divided by 6). When analyzed against time to progression for all evaluable patients, a statistically significant relationship was detected with 5T4 antibody levels ($P < 0.01$) but not with MVA-specific antibody levels ($P = 0.49$). If the calculation was further refined by analyzing only those patients who mounted a 5T4-specific antibody response, the relationship was stronger still ($P < 0.0001$).

Overall patient survival was modeled in a similar way using 5T4- and MVA-specific antibody levels as predictors; this yielded P values of 0.08 and 0.92, respectively. However, the magnitude of the 5T4-specific antibody response was found to be a predictor of improved patient survival ($P < 0.05$) within the 5T4 antibody responders. No relationships were detected when either survival or time to progression was analyzed against age of patient, sex, and circulating CEA levels or tumor burden at trial entry. No substantive correlations were found when the four predictors (5T4 antibody, MVA antibody, circulating CEA levels, and tumor burden) were plotted against each other in pairs and Spearman's correlation coefficient was calculated.

Discussion

The results presented here represent the first time in which a recombinant virus delivering the tumor antigen 5T4 has been tested in patients. The vaccine was shown to be safe and well tolerated at all doses and administration routes and the induction of an immune response against the self-protein was not associated with any deleterious autoimmune reactions. Phase I clinical studies traditionally recruit patients with metastatic disease, who are likely to have received significant prior treatment. Both of these factors may affect the individual's ability to mount effective antitumor immune responses. Indeed, it has previously been shown that cancer patients may show defects in the functioning of their T cells (20) or dendritic cells (21). Despite such compounding factors, 5T4-specific cellular and/or humoral immune responses were induced in the majority of patients (16 of 17; 94%) following TroVax immunization, which is encouraging compared with many other cancer immunotherapy trials (22).

Although the use of peripheral blood mononuclear cells to monitor immune responses enables only a snap shot of events which occur in the periphery to be determined, the number of sampling time points included in this study enabled some insights into the kinetics of both vector- and tumor-specific systemic responses. In the majority of patients, a single TroVax immunization was sufficient to induce potent MVA-specific cellular and humoral responses. In contrast, a 5T4-specific immune response was usually only evident following two or three vaccinations, suggesting that more than one injection is required to break tolerance to this self antigen. Both cellular and, to a lesser extent, humoral responses were relatively transient. This may reflect the need for continued boosting of the immune response in the presence of the tolerizing environment of the tumor or simply that responses detected in the periphery do not accurately mirror those occurring at the tumor site.

It is well documented that potent antivector immune responses can limit the utility of some viral vectors which need to be used more than once (e.g., homologous prime-boost regimens). Despite such potentially deleterious factors, it has previously been shown that the prior exposure of mice to

Table 2. 5T4-specific antibody titers in all per protocol patients measured at time points up to 52 weeks post primary immunization

Patient no.	Dose	Time point (wk)														
		0	2	4	6	8	10	12	16	18	20	24	30	32	34	36	52
101	1×	≤10	≤10	≤10	≤10	≤10	≤10	≤10	N/A	N/A	N/A	≤10	N/A	N/A	N/A	≤10	≤10
102	1×	≤10	≤10	≤10	**80**	**40**	**160**	**80**	**40**	N/A	≤10	≤10	**20**	**20**	≤10		
103	1×	≤10	≤10	≤10	≤10	≤10	≤10	≤10	≤10	N/A	N/A	≤10	≤10	**10**	≤10	≤10	
104	1×	≤10	≤10	≤10	≤10	≤10	**80**	**160**	≤10	N/A	≤10	**80**					
201	5×	≤10	≤10	≤10	≤10	≤10	≤10	≤10									
202	5×	≤10	≤10	≤10	≤10	≤10	≤10										
204	5×	≤10	≤10	≤10	**160**	**320**	**40**	≤10	≤10	N/A	≤10	≤10	**20**	N/A	≤10	≤10	≤10
205	5×	≤10	≤10	≤10	**320**	**40**	**80**	**80**	**80**	**40**	≤10	≤10	≤10	N/A	N/A	≤10	≤10
303	10×	≤10	≤10	≤10	≤10	≤10	**10**	≤10	N/A	≤10							
305	10×	≤10	≤10	≤10	**20**	**80**	**80**	**20**	≤10	≤10	≤10						
307	10×	≤10	≤10	≤10	**10**	≤10	≤10	≤10	≤10								
308	10×	≤10	≤10	≤10	**80**	**40**	**80**	**80**	≤10	≤10	≤10	**80**					
401	2×	≤10	≤10	≤10	**40**	**40**	**40**	**40**	≤10	≤10	≤10	≤10	N/A	N/A	N/A	≤10	≤10
402	2×	≤10	≤10	≤10	≤10	≤10	≤10	≤10	**10**	≤10	≤10						
404	2×	≤10	≤10	≤10	**80**	**80**	**80**	**80**	**40**	**20**	**20**	**80**	≤10	N/A	N/A	≤10	≤10
406	2×	≤10	≤10	≤10	≤10	**40**	≤10	≤10									
407	2×	≤10	≤10	**40**	≤10	≤10	≤10	≤10	**20**								

NOTE: Positive antibody titers are tabulated in bold font and underlined.
Abbreviations: N/A, samples were not available; blank cells, blood samples were not available due to patient withdrawal.

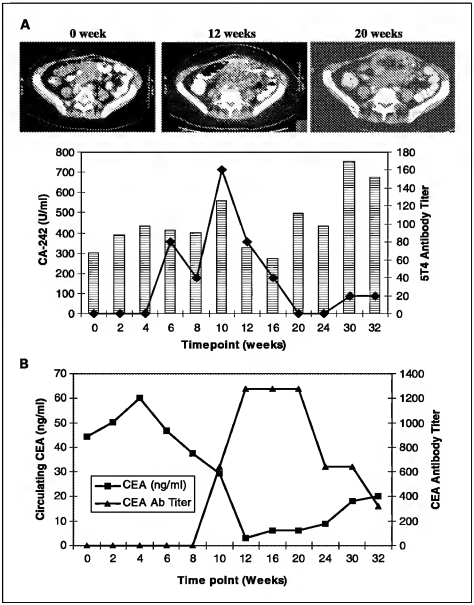

Fig. 2. Analysis of clinical and immunologic responses in patient 102. *A*, 5T4-specific antibody titer (*line*), circulating levels of plasma CA-242 (*hashed columns*), and CT scan images at weeks 0, 12, and 20. *B*, circulating levels of plasma CEA alongside the corresponding CEA antibody titer at the same time points.

vaccinia virus did not have a detrimental effect on the generation of an immune response to 5T4 delivered by TroVax (23). In the clinical setting, we have shown that a potent MVA-specific immune response is induced. This is encouraging when considering MVA as a possible smallpox vaccine candidate, but could be detrimental to the continued boosting of 5T4-specific responses in the cancer immunotherapy setting. Despite the presence of high levels of MVA neutralizing antibodies after just two TroVax vaccinations, 5T4-specific cellular and humoral responses were usually only evident after two or three immunizations and could be boosted after the fourth and fifth immunizations in some patients. Although the sample size was small, the group receiving the highest dose of TroVax (5×10^8 pfu) and those vaccinated i.d. seemed to have higher levels of neutralizing antibodies but no marked decrease in either the incidence or magnitude of 5T4-specific immune responses. Two patients (103 and 407) presented with detectable levels of neutralizing antibody at trial entry, despite which 5T4-specific immune responses were detected after TroVax vaccination. Furthermore, several patients who had a low level of MVA neutralizing antibodies (101 and 201) compared with other patients failed to show 5T4-specific antibody responses, suggesting that other factors affect the induction of immune response to the tumor antigen. Although it was impossible to conclude that the presence of neutralizing antibodies did not affect the magnitude of 5T4-specific immune responses induced, there was no correlation between high neutralizing antibody titers and low tumor antigen responses in this study.

This trial analyzed the ability of TroVax to induce 5T4-specific immune responses when delivered in increasing doses and via either i.m. or i.d. routes. Although this trial was not powered to allow conclusions to be drawn from differences in immune read-out between each group, there was no striking evidence that vaccination either i.m. or i.d. or at a 1×, 2×, 5×, or 10× dose induced a substantially stronger or long-lived immune response.

The retrospective statistical analysis aimed to identify potential correlations between a number of different variables and time to disease progression or overall patient survival. The only variable which showed a significant relationship with both increased time to progression and enhanced patient survival was found to be the 5T4-specific antibody titer. The relationship with time to progression was highly significant ($P < 0.01$ for all evaluable patients and $P < 0.0001$ for those patients who sero-converted). The interpretation of the relationship with overall patient survival was complicated by the fact that a number of patients who were withdrawn from the trial subsequently received additional therapies (e.g., 5-fluorouracil + Irinotecan). The relationship between overall survival and 5T4 antibody titer yielded moderate P values which are very encouraging in a small phase I/II study, but should be interpreted with some caution for several reasons. First, the study is small; therefore, the P value is vulnerable to such external factors as data errors or an unusual subject mix. Second, the decision to analyze the data, especially to stratify by responder/nonresponder to 5T4, was made after inspection of the data. Finally, in an uncontrolled trial, it is only possible to show association, not causation. There could conceivably be some third unmeasured factor which results in both a higher 5T4 antibody response (in those that do respond) and a higher chance of survival. This question can only be addressed by a randomized controlled trial. Despite such caveats, it is

Table 3. MVA-specific total immunoglobulin G and neutralizing antibody titers

Patient no.	Dose group	Prevaccinated	Time point (wk)					
			0	4	8	12	24	52
101	1×	Yes	**8,000**	**8,000**	8,000	8,000	4,000	8,000
			–	+	+	+	ND	ND
102	1×	Yes	<4,000	**16,000**	16,000	32,000	16,000	N/A
			–	+	++	++	ND	N/A
103	1×	Yes	<4,000	**32,000**	16,000	32,000	8,000	N/A
			+	++	+++	++	ND	N/A
104	1×	No	<4,000	**4,000**	8,000	8,000	<4,000	N/A
			–	–	++	+	ND	ND
201	5×	No	<4,000	**4,000**	16,000	8,000	N/A	N/A
			–	+	–	–	N/A	N/A
202	5×	No	<4,000	<4,000	<4,000	N/A	N/A	N/A
			–	++	+	++	N/A	N/A
204	5×	No	**4,000**	**8,000**	16,000	32,000	16,000	16,000
			–	+	+	++	ND	ND
205	5×	Yes	<4,000	**4,000**	<4,000	<4,000	<4,000	<4,000
			–	–	–	+	ND	ND
303	10×	Yes	<4,000	**32,000**	16,000	32,000	N/A	N/A
			–	++	+++	+++	N/A	N/A
305	10×	No	<4,000	**4,000**	8,000	16,000	N/A	N/A
			–	+	++	+++	N/A	N/A
307	10×	No	<4,000	**4,000**	32,000	128,000	N/A	N/A
			–	+	+++	+++	N/A	N/A
308	10×	Yes	<4,000	**128,000**	128,000	128,000	N/A	N/A
			–	+++	+++	+++	N/A	N/A
401	2×	No	<4,000	**32,000**	32,000	64,000	16,000	<4,000
			–	++	++	++	ND	ND
402	2×	Yes	**4,000**	**8,000**	16,000	16,000	N/A	N/A
			–	+	++	++	N/A	N/A
404	2×	Yes	<4,000	**8,000**	16,000	8,000	32,000	8,000
			–	++	++	++	ND	ND
406	2×	Yes	<4,000	<4,000	<4,000	<4,000	N/A	N/A
			–	–	++	++	N/A	N/A
407	2×	Yes	<4,000	<4,000	**128,000**	**128,000**	N/A	N/A
			++	++	+++	+++	N/A	N/A

NOTE: For each patient, the MVA-specific immunoglobulin G antibody titer is tabulated in the top row and the neutralizing antibody titer listed in the lower row as negative (–) or positive (+, ++, or +++). The dose group and prior smallpox vaccination status are tabulated for each patient. Figures in bold represent positive immunoglobulin G antibody responses at that time point and those in bold and underlined are positive compared with preinjection levels. Neutralizing antibody titers are defined as <1:20 (–), ≥1:20 (+), ≥1:100 (++), or ≥1:500 (+++).
Abbreviation: ND, result was not determined.

Table 4. Proliferative responses of patients' peripheral blood mononuclear cells in response to stimulation with 5T4 protein

Patient no.	Dose	Time point (wk)															
		0	2	4	6	8	10	12	16	18	20	24	30	32	34	36	52
101	1×	**2**	1.2	0.9	1.8	1.2	**0.9**	0.5	N/A	N/A	N/A	1	N/A	N/A	N./A	**0.8**	1.5
102	1×	1	1.9	1.8	0.5	**3.1**	**3.5**	**4.6**	0.9	N/A	0.9	**2.7**	1.9	**3.5**	0.6		
103	1×	0.9	**3.3**	0.8	1.4	1.4	**5.7**	**1**	1.1	N/A	N/A	1.2	0.9	1.4	1.1	0.5	
104	1×	1.9	**1**	1	1.1	**3.7**	**3.4**	0.9	0.7	N/A	0.9	**2.8**					
201	5×	1	1	0.4	0.8	0.5	0.8	1.7									
202	5×	0.3	1.2	0.7	0.5	0.3	0.4										
204	5×	0.6	1.4	0.3	0.8	**4.1**	0.8	0.8	0.4	N/A	1.3	1.3	1.4	N/A	1.3	**5.9**	0.4
205	5×	**5.5**	**2.6**	**9.5**	0.5	1.2	**15**	0.6	0.7	**2.8**	0.5	0.6	**2.3**	N/A	N/A	0.8	**2**
303	10×	**2.2**	1	0.8	0.5	0.6	1	0.7	N/A	0.7							
305	10×	0.3	1.7	**15.8**	0.5	0.9	0.6	0.8	0.7	1.8	0.5						
307	10×	**6.5**	1.6	**5.5**	0.4	0.7	**2.7**	1.1	0.8								
308	10×	0.8	**4.8**	**3.7**	1.2	0.6	0.8	0.9	0.6	**2.8**	1.2	0.5					
401	2×	1.3	0.7	0.9	0.3	1	1.2	0.6	0.7	1.3	0.5	1.7	N/A	N/A	N/A	1.6	0.8
402	2×	1.4	0.9	1	1.9	0.8	0.6	**5.8**	1.2	0.9	1.2						
404	2×	1	0.5	0.8	**3.9**	0.8	1.2	0.5	1.1	1	**2.8**	1	1	N/A	N/A	**2**	**5.2**
406	2×	0.7	0.8	0.3	1.3	1.9	1.3	0.6									
407	2×	0.4	0.5	**2.1**	1.8	1	1	0.8	0.8								

NOTE: Results are expressed as a stimulation index. A stimulation index ≥2 (indicated by bold text) is considered to be a positive response at that time point. Proliferative responses which are positive (stimulation index ≥2) and at least 2-fold greater than the preinjection (−2 or 0 week) responses are considered to be positive due to vaccination and are shown in bold and underlined. Blank cells, blood samples were not available due to patient withdrawal.

important to note that no relationship was found between enhanced patient survival and the induced MVA-specific antibody response. If improved survival was simply a function of the general health status and immune competence of these patients, it is likely that the antibody response to both 5T4 and MVA would show an association with survival. Furthermore, other indicators of disease status, the magnitude of circulating CEA, and the tumor burden at trial entry failed to show

Table 5. Summary immunologic and clinical data

Patient no.	No. injections	Antibody response		Proliferative response			Disease status (mo)					
		5T4	MVA	5T4 protein	5T4 peptide	MVA	3	6	9	12	15	18
101	3	No	Yes	No	No	No	PD	PD	PD	PD	—	—
102	4	Yes	Yes	Yes	Yes	Yes	PD	PD	—	—	—	—
103	5	Yes	Yes	Yes	Yes	Yes	PD	PD	PD	—	—	—
104	4	Yes	Yes	Yes	Yes	Yes	SD	PD	—	—	—	—
201	3	No	Yes	No	Yes	Yes	PD	—	—	—	—	—
202	3	No	No	No	Yes	No	PD	—	—	—	—	—
204	5	Yes	Yes	Yes	Yes	Yes	SD	SD	SD	SD	SD	SD
205	5	Yes	Yes	Yes	Yes	Yes	SD	SD	PD	—	—	—
303	3	Yes	Yes	No	No	Yes	PD	—	—	—	—	—
305	5	Yes	Yes	Yes	Yes	Yes	PD	PD	—	—	—	—
307	4	Yes	Yes	Yes	Yes	Yes	PD	—	—	—	—	—
308	5	Yes	Yes	Yes	Yes	Yes	PD	PD	—	—	—	—
401	5	Yes	Yes	No	Yes	Yes	SD	PD	PD	PD	—	—
402	5	Yes	Yes	Yes	Yes	Yes	PD	PD	—	—	—	—
404	5	Yes	Yes	Yes	Yes	Yes	SD	SD	PD	PD	—	—
406	3	Yes	No	No	Yes	Yes	PD	—	—	—	—	—
407	4	Yes	Yes	Yes	Yes	Yes	PD	—	—	—	—	—

NOTE: The table details the number of TroVax injections received by each patient and tabulates whether a positive 5T4 (protein or peptide) or MVA-specific immune response was induced in each patient following vaccination. The disease status of each patient determined by CT scan analysis and assessed by standard WHO criteria is tabulated alongside immunologic responses. Disease status is tabulated as progressive disease (PD) or stable disease (SD).

correlations with survival. Overall, the relationship between 5T4 antibody titer and improved patient survival represents an encouraging early efficacy result. The result is particularly interesting because 5T4-specific antibodies were shown to be essential for protection against tumor challenge in a murine 5T4 tumor model (16). Moreover, it has previously been shown that a chimeric 5T4-specific single-chain antibody can direct antibody-dependent, cell-mediated cytotoxicity of human tumor cell lines (24).

In summary, the present study shows the safety and immunogenicity of TroVax delivered via i.m. and i.d. routes of administration. Early indications of clinical benefit which correlate with 5T4-specific antibody responses are encouraging and support the further development of TroVax as a potential vaccine for cancer immunotherapy.

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